

# Allocation of $^{14}\text{C}$ assimilated in late spring to tissue and biochemical stem components of cork oak (*Quercus suber* L.) over the seasons

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Carbon distribution in the stem of 2-year-old cork oak plants was studied by  $^{14}\text{CO}_2$  pulse labeling in late spring in order to trace the allocation of photoassimilates to tissue and biochemical stem components of cork oak. The fate of  $^{14}\text{C}$  photoassimilated carbon was followed during two periods: the first 72 h (short-term study) and the first 52 weeks (long-term study) after the  $^{14}\text{CO}_2$  photosynthetic assimilation. The results showed that  $^{14}\text{C}$  allocation to stem tissues was dependent on the time passed since photoassimilation and on the season of the year. In the first 3 h all  $^{14}\text{C}$  was found in the polar extractives. After 3 h, it started to be allocated to other stem fractions. In 1 day,  $^{14}\text{C}$  was allocated mostly to vascular cambium and, to a lesser extent, to primary phloem; no presence of  $^{14}\text{C}$  was recorded for the periderm. However, translocation of  $^{14}\text{C}$  to phellem was observed from 1 week after  $^{14}\text{CO}_2$  pulse labeling. The phellogen was not completely active in its entire circumference at labeling, unlike the vascular cambium; this was the tissue that accumulated most photoassimilated  $^{14}\text{C}$  at the earliest sampling. The fraction of leaf-assimilated  $^{14}\text{C}$  that was used by the stem peaked at 57% 1 week after  $^{14}\text{CO}_2$  plant exposure. The time lag between C photoassimilation and suberin accumulation was  $\sim 8$  h, but the most active period for suberin accumulation was between 3 and 7 days. Suberin, which represented only 1.77% of the stem weight, acted as a highly effective sink for the carbon photoassimilated in late spring since suberin specific radioactivity was much higher than for any other stem component as early as only 1 week after  $^{14}\text{C}$  plant labeling. This trend was maintained throughout the whole experiment. The examination of microautoradiographs taken over 1 year provided a new method for quantifying xylem growth. Using this approach it was found that there was more secondary xylem growth in late spring than in other times of the year, because the calculated average cell division time was much shorter.

**Keywords:** carbon assimilation, C-14 labeled compounds, microautoradiography, *Quercus suber*, suberin.

## Introduction

Cork oak (*Quercus suber* L.) is an evergreen oak species of the Mediterranean sclerophyll forests, where it occupies  $\sim 2.28$  Mha, 55% of which are located in the Iberian Peninsula (APCOR 2011). Cork oak areas are specific multiple use systems that include forest, agricultural and animal production, and have a high ecological value (see Aronson et al. 2009).

In the Iberian Peninsula, the growing season of cork oak lasts from approximately April to October, depending on rainfall and temperature (Oliveira et al. 1994, González-Pérez et al. 2008, Pinto et al. 2011). The highest growth rate occurs at the end of spring due to a high photosynthetic activity and water availability (Vaz et al. 2010) with the use of accumulated carbohydrate reserves as common to sclerophyll Mediterranean species (Mooney et al. 1974, Larcher and Thomaser-Thin

1988, Cerasoli et al. 2004). Correspondingly, tree radial growth presents a clear seasonal pattern. It peaks in June–July in cork oak stands grown in the western central part of Portugal; there, the period of dormancy lasts from November to February (Costa et al. 2002, 2003). During this period cork oak, as an evergreen tree, has the possibility of photosynthesizing. Thus, studies conducted with isotopic tracers (Cerasoli et al. 2004) show that winter assimilates are retained within the plant and later on contribute to sustain the seasonal flush growth of cork oak. Cerasoli et al. (2004) focused on winter assimilates, and to the best of our knowledge, their work is the only study that investigated the allocation of photoassimilated C and the following seasonal changes in cork oak.

Cork is the external part of the bark periderm of cork oak that covers the tree stem and branches. It is differentiated by the phellogen, the bark meristematic layer that produces the living phelloderm cells on the inside, and the phellem (suber or cork) cells on the outside that suberify and die. Phellogen formation and activity starts during the second year of plant growth and the cork layer becomes clearly visible after 5 years (Graça and Pereira 2004). On the tree, cork functions as a barrier against biological and abiotic attacks, such as fire, due to the protective properties resulting from its regular and compact cellular structure and cell wall chemical composition. Literature on topics related to cork from cork oak is extensive; it is worth mentioning the review by Pereira (2007).

Chemical composition of cork has been widely studied (see Pereira 1988, Silva et al. 2005, Jové et al. 2011). As stated in the review by Silva et al. (2005), cork composition depends on a number of factors, like the geographical origin, the climate and soil conditions or the type of cork. Jové et al. (2011) studied the composition of the different layers of cork oak bark (belly, cork and back) and they found that suberin content ranged from 21.1% (back layer of a sample from Cáceres, Spain) to 53.1% (belly layer of a sample from Ciudad Real, Spain). Silva et al. (2005) mentioned a range between 33 and 50% from literature data. Other cork components are lignin (14.8–31%), holocellulose (2.3–33.6%), extractives (7.3–20.4%) and ash (0.4–3.3%) (values reported by Jové et al. 2011). Waxes, which are important constituents of the extractives, represent between 4.67 and 8.14%, according to Conde et al. (1999). Recent contributions to the knowledge of the phenolic fraction of the extractives are those by Santos et al. (2010) and Fernandes et al. (2011).

Among the chemical components of cork, suberin has been recognized as a major component. Suberin is usually described as a heteropolymer with polymeric aliphatic and associated aromatic materials, deposited and immobilized in the plant cell wall (Ranathunge et al. 2011). Main monomers are  $\omega$ -hydroxyacids ( $\omega$ -OH-acids) and  $\alpha,\omega$ -dicarboxylic acids ( $\alpha,\omega$ -diacids) of C16 to C30 chain length that are esterified to glycerol and cross-esterified, and the aromatic zone includes

ferulic acid (Graça and Pereira 1997, 2000). The 3D chemical structure of suberin is still largely unknown and there are many gaps regarding its biosynthetic pathway (Pollard et al. 2008). Progress made in elucidating suberin biosynthesis has been recently reviewed by Ranathunge et al. (2011). They also discussed the physiological role of suberin in plant tissues, which is associated with its persistent and hydrophobic nature. However, they concluded that many issues relative to suberin remain unresolved. According to the literature, suberin is an interesting polymer that needs to be investigated. Some authors highlight the potential of suberin for the synthesis of polymeric materials and chemicals (Cordeiro et al. 1998, Pinto et al. 2009).

In the present study, the distribution of C assimilated in late spring among the various tissues and biochemical components of cork oak stem was investigated. Working hypothesis was that the allocation of C photoassimilated by cork oak in the growing season would vary with translocation time, while suberin, major component of cork, would act as a strong sink. Specific objectives of this work were: (i) to understand how much C from leaf assimilation is needed to ensure stem growth, (ii) to assess changes in leaf-assimilated C partitioning over different periods of time, concerning stem chemical components and tissues, (iii) to determine the time lag between C assimilation and suberin accumulation and (iv) to understand how long the stem growth persists during the seasons.

To meet our objectives, an isotopic tracer experiment was conducted that involved  $^{14}\text{C}$  pulse labeling of cork oak saplings in late spring. The fate of  $^{14}\text{C}$  photoassimilated carbon was followed during two periods: the first 72 h (short-term study) and the first 52 weeks (long-term study) after the  $^{14}\text{CO}_2$  photosynthetic assimilation, in order to cover the annual growth cycle. The use of  $^{14}\text{C}$  radioactive isotope instead of the  $^{13}\text{C}$  stable isotope was essential for tracing histologically the fate of labeled assimilates. Microautoradiography is made possible by the fact that  $^{14}\text{C}$  emits  $\beta$ -radiation, so  $^{14}\text{C}$ -labeled tissues can be visualized with a radiation-sensitive silver emulsion and the image produced processed using photographic procedures. For methodological reasons these techniques are restricted to small plants – 2-year-old cork oak saplings in this study – however, as shown in the literature, these techniques can provide very interesting information.

## Materials and methods

### Location and plant material

This study was carried out in the experimental fields of the College of Agricultural Engineering of Madrid, Spain. A total of 56 plants of 2-year-old cork oak (*Quercus suber* L.) were grown for this work. The plants were raised, from acorns collected in a natural cork oak stand located in 'El Pardo' (Madrid, Spain), in



polyethylene containers of 10 l capacity and 25 cm diameter, filled with a homogeneous substrate of vegetal soil and peat (3 : 1). The seeds germinated in spring; seedlings were then grown under non-limiting water conditions until the age of 2 years, when this study was initiated. The average height and dry weight of the individual plants were  $92.5 \pm 9.6$  cm and  $39.1 \pm 11.4$  g (total biomass), respectively; stem represented 20% of the plant dry weight.

### Experiment design

The experiment was designed as a 1-year follow-up study of the chemical composition and the histology of the stem of  $^{14}\text{C}$ -pulse-labeled cork oak plants. The  $^{14}\text{CO}_2$  photosynthetic assimilation was done in June, which is within the period of most active growth of cork oak according to the literature (Oliveira et al. 1994, Fialho et al. 2001, Pinto et al. 2011).

Plants were individually exposed to a pulse of  $^{14}\text{CO}_2$  and after that, the allocation of  $^{14}\text{C}$  to stem chemical components was determined throughout two periods of time, 72 h (short-term study) and 52 weeks (long-term study), respectively. Four plants were used in each sampling. The microautoradiographic study was carried out in parallel with the long-term chemical study. The work schedule for this study involved samplings at 1, 3, 8, 24 and 72 h after  $^{14}\text{CO}_2$  exposure and subsequently at 1, 2, 3, 4, 6, 8, 26 and 52 weeks.

### $^{14}\text{C}$ plant labeling

The day before  $^{14}\text{C}$  labeling, the plants were prepared by removing dead leaves and weeds and by covering the container's surface with a black polyethylene film. The plants were left in darkness for 12 h prior to exposure to  $^{14}\text{CO}_2$  to avoid any possible depression of photosynthesis during  $^{14}\text{CO}_2$  assimilation (Tenhunen et al. 1984, Faria et al. 1996).

Methodology used in this work for  $^{14}\text{C}$  plant labeling followed relevant methods of  $^{14}\text{C}$  pulse labeling reported in the literature (see Fernández et al. 2003, Lockhart et al. 2008). Plant exposure to  $^{14}\text{CO}_2$  was carried out in a cylindrical chamber made of transparent methacrylate (70 cm high and 15 cm in diameter) covered at the top by a transparent polyethylene bag (60 cm  $\times$  38 cm), which made a total volume of  $\sim 15$  l. Each plant was introduced into the chamber separately, and the inferior rim of the cylinder lightly pressed onto the polyethylene film which covered the container soil surface. To ensure complete sealing, water was added in order to form a hydraulic seal  $\sim 2$  cm high between the container and the external chamber wall (Figure 1). The whole cork oak plant + exposure chamber was placed inside a laboratory safety cabinet prior to the  $^{14}\text{CO}_2$  assimilation. Next, the safety cabinet was equipped with a mixed light source from two lamps (a sodium vapor lamp Philips SON/T 400 W, Madrid, Spain and a metal halide lamp Osram Powerstar HQI-T-400W/DH, Munich, Germany). Photosynthetic photon flux density (photosynthetically active

radiation, PAR) intercepted by the leaves of the cork oak plant in the cabinet ranged from  $975 \pm 39 \mu\text{E m}^{-2} \text{ s}^{-1}$  at 80 cm height (25 cm from the light source) to  $270 \pm 24 \mu\text{E m}^{-2} \text{ s}^{-1}$  at 30 cm height (75 cm from the light source).

The total radioactivity introduced into the chamber in each experiment was  $1.17 \times 10^6$  Bq, which was equivalent to 78 Bq  $\text{ml}^{-1}$  initial radioactive concentration in the air. The preparation and introduction of  $^{14}\text{CO}_2$  into the chamber was as follows:  $^{14}\text{CO}_2$  was prepared by the reaction of 2 N lactic acid with  $\text{NaH}^{14}\text{CO}_3$  with a specific activity of  $2.06 \times 10^6$  Bq  $\mu\text{mol}^{-1}$ ; then it was injected into the chamber using a 100 ml syringe with a two-way valve (Figure 1). A 12 V minifan was placed inside the chamber to ensure homogenization of the air containing  $^{14}\text{CO}_2$ . The amount of  $\text{CO}_2$  added was 0.568  $\mu\text{mol}$  (as  $^{14}\text{CO}_2$ ), while the amount of air  $\text{CO}_2$  in the chamber was 261.2  $\mu\text{mol}$  (390  $\text{ml m}^{-3}$ , determined by infrared gas analyser), so the addition of  $^{14}\text{CO}_2$  was not significant for the air  $\text{CO}_2$  concentration.  $^{14}\text{CO}_2$  content of the atmosphere inside the chamber was monitored by sampling and scintillation counting (Warembourg and Kummerow 1991). Plants were maintained in these conditions until 95%  $^{14}\text{CO}_2$  absorption ( $\sim 2$  h), when the  $^{14}\text{C}$  labeling was considered as finalized. Before opening the chambers, the air inside was re-circulated through a soda lime trap to fully remove any  $^{14}\text{CO}_2$  and prevent radioactive contamination outside of the chambers. A maximum of 18 plants were labeled per day (in groups of 4–6 plants per labeling experiment). Labeling of all plants for this work was completed in three consecutive days.

### Plant conditions for $^{14}\text{C}$ translocation

After  $^{14}\text{C}$  assimilation, the cork oak plants were placed outdoors under the climate conditions pertaining to the Polytechnic University Campus (latitude  $40^\circ 26' 36'' \text{N}$ , longitude  $03^\circ 44' 18'' \text{W}$ , altitude 595 m), which correspond to a continental Mediterranean climate. Soil moisture was maintained at  $>60\%$  field capacity; a Time Domain Reflectometry (TDR) probe was used to control soil humidity.

The mean, maximum and minimum temperatures recorded during the experiment are shown in Figure 2. Temperatures followed the normal pattern for the site. The absolute minimum temperature ( $-8^\circ \text{C}$ ) was recorded in February, and the absolute maximum temperature ( $43^\circ \text{C}$ ) in July.

### Sampling and sample preparation

Samplings were performed at random taking four plants each time; this was done 1, 3, 8, 24 and 72 h after plant  $^{14}\text{CO}_2$  exposure for the short-term study and 1, 2, 3, 4, 6, 8, 26 and 52 weeks after  $^{14}\text{C}$  photoassimilation for the long-term study.

The preparation of stem samples was the same for every plant. The central part of the plant stem was carefully cut off and used for  $^{14}\text{C}$  quantification (short-term and long-term studies) and for histological preparation (microautoradiographic

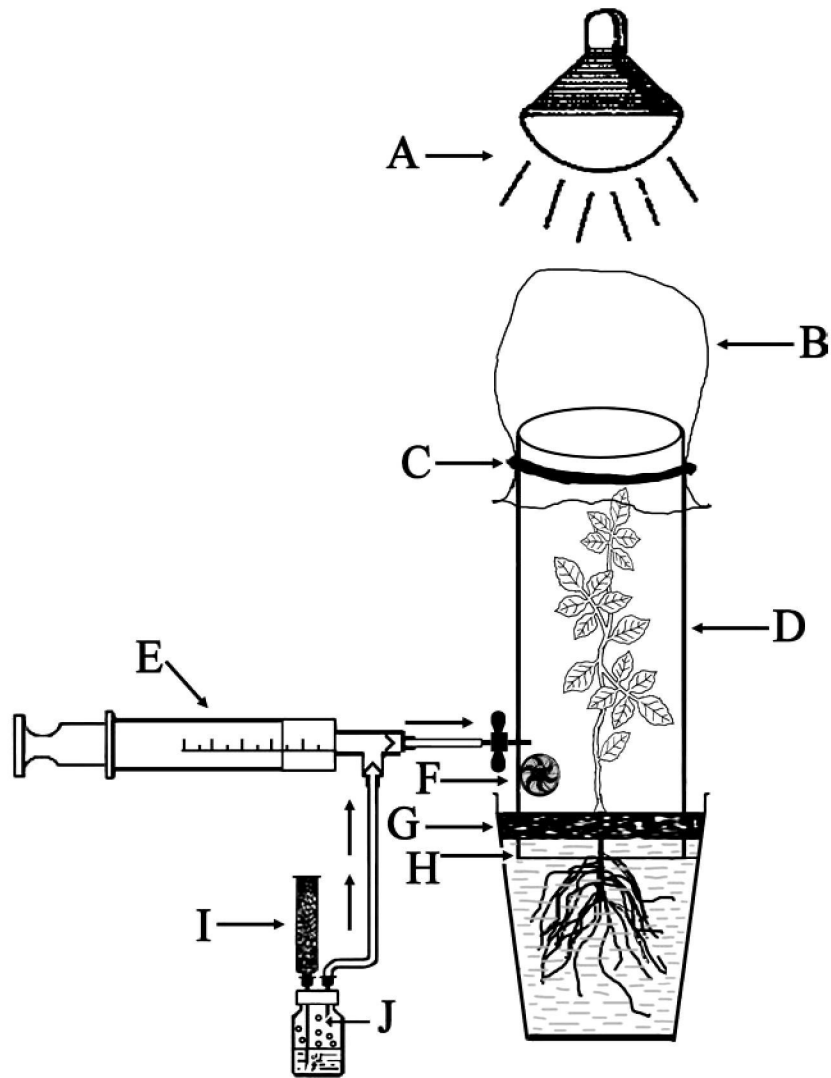


Figure 1. Design of the plant chamber system for  $^{14}\text{C}$  pulse labeling. A, light source; B, polyethylene film; C, elastic band; D, methyl methacrylate cylinder; E, syringe; F, minifan; G, hydraulic closure; H, polyethylene film cover; I, fiberglass filter; J,  $^{14}\text{CO}_2$  gas

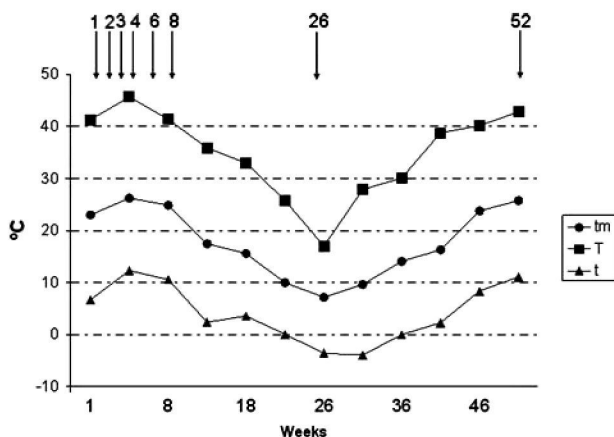


Figure 2. Mean ( $t_m$ ), maximum ( $T$ ) and minimum temperatures ( $t$ ) recorded during the experiment ( $^{\circ}\text{C}$ ). The arrows show the sampling weeks.

study). Basal and apical fractions of the stem (~10 cm in length) were discarded. Afterwards, each stem sample was carefully cut into transverse fractions of 1 cm length. The samples for the chemical study were further cut into small pieces and then they were oven-dried at  $80^{\circ}\text{C}$  for 24 h. For the microautoradiographic study, the sample preparation involved standard histological procedures of fixation and inclusion. Histological fixation was achieved by immersion of 3–4 mm fresh cross sections of the stem samples in 3% glutaraldehyde solution for 24 h. Afterwards, the sections were rinsed with water for 1 h and placed in a PEG (polyethylene glycol 2000, Merck, Darmstadt, Germany)–water 1:5 solution at  $60^{\circ}\text{C}$  until water evaporation. Polyethylene glycol-embedded sections were then transferred to square frames filled with melted PEG and left at room temperature until PEG solidification to complete the inclusion in PEG of the stem sections.



### Chemical fractioning

Chemical fractioning was performed using the methodology described by Pereira (1988) for cork, which involves sequential extraction procedures, after the necessary adaptation to work with radioactive tracers. Two replications of 500–850 mg each (oven-dried matter) were used per plant.

After each extraction step, the liquid fraction was frozen at  $-8^{\circ}\text{C}$  for subsequent measurement of radioactivity and the solid residue was oven-dried at  $60^{\circ}\text{C}$  for 24 h. The solid residue was then weighed and the next step in the extraction process started, until total solubilization. The full sequence was as follows: (i) extraction with dichloromethane (25 ml) under reflux for 3 h for solubilization of waxes and surface lipids; (ii) extraction with absolute ethanol (25 ml) under reflux for 4 h for solubilization of alcohol-soluble phenols and carbohydrates; (iii) extraction with water (25 ml) under reflux for 6 h for solubilization of tannins and water-soluble carbohydrates; (iv) hydrolysis with 3% sodium methoxide in methanol (50 ml) under reflux for 3 h for solubilization of suberin and more deeply located lipids (henceforth, components solubilized at this step are referred to as suberin) (Marques and Pereira 1987); (v) hydrolysis with 1% v/v sulfuric acid (25 ml) at  $121^{\circ}\text{C}$  for 10 min in an autoclave for solubilization of hemicelluloses and the most labile cellulosic fractions (Browning 1963, Han 1983); (vi) hydrolysis with 3% v/v sulfuric acid (25 ml) at  $121^{\circ}\text{C}$  for 1 h in an autoclave for solubilization of amorphous cellulose fractions (Immergut 1963, Han 1983); (vii) hydrolysis with 72% v/v sulfuric acid (3 ml) at ambient temperature for 1 h, dilution in water to a 3% solution and autoclaving at  $121^{\circ}\text{C}$  for 1 h for solubilization of crystalline cellulose (Browning 1963, Pereira 1988); and (viii) hydrolysis with 25 ml of a water solution (1 l) of nitric acid (90 ml) and acetic acid (732 ml) under reflux for 45–60 min for solubilization of lignin (Pereira 1988).

The amount of each component extracted was found by measuring the difference in mass of the dried solid residue before and after each step.

### Measurement of the radioactivity in the extracts

The radioactivity of the liquid extracts obtained after each extractive or reactive step was measured in a Beckman LS-580 liquid scintillation counter (LSC) using Ready Micro scintillant (Beckman Instruments, Inc., Fullerton, CA, USA) for neutral and basic extracts, and Ready Gel scintillant (Packard/Beckman) for the acid extracts. Aliquots of 0.1 ml extract were taken and transferred to scintillation minivials; then, 3 ml scintillant per minivial was added and the samples were measured for radioactivity in the LSC. All measurements were carried out with four replications. The radioactive concentration in each minivial was expressed in  $\text{Bq ml}^{-1}$  according to the values of radioactivity recorded by the scintillation counter. The total radioactivity of each liquid extract was calculated from

the respective radioactive concentration and extract volume. From that value of total radioactivity, the specific activity ( $\text{Bq mg}^{-1}$ ) of each group of chemical components—solubilized by extraction or hydrolysis—was calculated taking into account the results of mass quantification.

### Microautoradiographic technique

Sample holders were cleaned with a chromium–sulfuric mixture (30 g potassium dichromate in 1 l 96% sulfuric acid) for 24 h, then washed in aqueous  $\text{NH}_4\text{OH}$  solution (1 l deionized water plus 30 drops of ammonia 30%), and dried in a chamber with filtered air for  $\text{NH}_3$  removal and stored in 96% ethanol. One day before use, the sample holders were coated with a thin gelatin film (5 g gelatin, 0.5 g  $\text{CrK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ , 100 ml water), which acts as a specific adhesive film for autoradiography, and is left to dry in a dark, dry chamber.

The stem sections included in PEG were cut with a sliding microtome (Shibuya Optical Co. Ltd, Tokyo, Japan) suitable for ligneous plant material, in order to make cross-sections  $\sim 10\text{ }\mu\text{m}$  thick. Prior to sectioning, and to help obtain a complete cross section, a transparent adhesive tape (Tesafilem® 106; Tesa, Hamburg, Germany) was placed on the sample that would stay stuck to the stem section during and after the cutting. Then, the cut sections were immersed in water for 5–10 s for PEG removal and left to dry at room temperature for 10 min. The stem sections and their supporting adhesive tape were carefully placed on the previously prepared sample holders with the tape upwards in order to stick the stem sections to the gelatin film that coated the sample holders. Then they were introduced into an oven at  $60^{\circ}\text{C}$  for 24 h to achieve complete adhesion of the stem section to the sample holder. Afterward they were immersed in acetone for 10 min to eliminate the tape, and rinsed with deionized water. Finally, they were introduced into a saturated solution of Sudan IV in 80% ethanol for 20 min, by which time the suberified cell walls were stained red.

Microautoradiographies were carried out using the ‘dipping’ methodology developed by Jofte and Warren (1955) after being adapted for use with lignocellulosic materials. After sample staining, the sample holders were washed with distilled water and placed in a dark chamber. There they were coated with a Kodak NTB-2 electron-sensitive emulsion of  $0.30\text{ }\mu\text{m}$  crystal diameter and grade 3 Ilford sensitivity (Fischer 1975). The samples were dried and kept for 8 weeks in complete darkness in order to obtain the reduction of the silver in the sensitive film by the  $\beta$ -radiation emitted by  $^{14}\text{C}$  in the stem tissues. After that, the film was developed by immersing the sample holders in 8 : 100 Kodak D-19 (Eastman Kodak Company, USA) aqueous solution at  $20^{\circ}\text{C}$  for 4 min; then the film was rinsed with deionized water, dipped into a 1 : 7 AGEFIX-AGFA solution (AGFA-GEVAERT, Belgium) for 10 min and further rinsed for 10 min. Later, one drop of gelatin–glycerol was put

on each stem section and finally, each of these was covered with a lamella.

Microscopic examination of microautoradiographs was performed by using an Olympus BH2 microscope (Olympus Corporation, Tokyo, Japan) equipped with a Sony Mod.DXC-107P (Sony Corporation, Tokyo, Japan) camera connected to a PC by means of an interface. On the whole, the number of microscopical preparations examined was 168 (7 dates  $\times$  4 plants per date  $\times$  6 stem sections). Photographs were taken at a resolution of 600 dpi.

### Analysis of results

Statistical analysis of the results of the chemical study was done using StatGraphics Plus 5.1 (Manugistics, Inc., Rockville, USA). Analysis of variance was done separately for the results of the short-term study (period comprised between 1 and 72 h after the  $^{14}\text{CO}_2$  plant exposure) and the long-term study (period comprised between 1 and 52 weeks after the  $^{14}\text{CO}_2$  plant exposure). The differences were considered significant at the level of  $P < 0.05$ . When it was appropriate, means were compared using the Tukey test; whenever normality was not obtained, a non-parametric test (Kruskal–Wallis) was used.

Main events observed in the chemical study (long-term and short-term studies) and in the microautoradiographic study were compiled in order to infer a pattern of  $^{14}\text{C}$  allocation. Additionally, a quantitative method of xylem growth evaluation was developed from the microautoradiographic study.

## Results

### Chemical fractioning of biomass

The proportion of chemical components did not change significantly ( $P \geq 0.05$ ) throughout the year. The major fraction was that of the lignocellulosic components which accounted for 81.4% of the total biomass on average, including lignin (25.4%), hemicelluloses (25.6%), amorphous cellulose (18.5%) and crystalline cellulose (11.9%). The soluble components represented 1.0 and 15.8%, respectively, for non-polar and polar extractives. Suberin amounted to 1.8% of the total biomass.

Biomass partitioning into chemical components showed that most stem biomass was allocated to xylem and phloem, and only to a much smaller extent to the periderm. Suberin is an indicator of the proportion of the periderm since it is only found in the phellem cells where it represents ~40–45% of the cell walls, the remaining being soluble materials and a lignocellulosic matrix (Pereira 1988, Graça and Pereira 2004). Therefore, a rough estimate of the periderm content in the stem biomass can be made using this proportion, giving ~5%.

### Translocation of photoassimilated $^{14}\text{C}$ in the first 72 h after labeling

Table 1 shows the specific radioactivity ( $\text{Bq mg}^{-1}$ ) measured in the cork oak stems and in their chemical fractions for the samplings performed in the period between 1 and 72 h after  $^{14}\text{CO}_2$  plant exposure. Radioactivity distribution (as % of total radioactivity obtained in each sampling) is also given in Table 1. The  $^{14}\text{C}$  that was assimilated by the leaves was already detected in the stem at low levels ( $< 1 \text{ Bq mg}^{-1}$ ) 1 h after plant exposure to  $^{14}\text{CO}_2$  but only in the ethanol and water-soluble components. Translocation of  $^{14}\text{C}$  increased during the first 3 h but remained restricted to the ethanol and water-soluble components (100% total radioactivity).

The radioactivity concentration in the stem continued to increase with time, reaching the values of 7.2, 9.3 and  $15.6 \text{ Bq mg}^{-1}$  after 8, 24 and 72 h, respectively. Also the  $^{14}\text{C}$  accumulation increased steadily in the ethanol–water-soluble components over time. Incorporation into the other components started after 8 h and increased subsequently, especially in suberin ( $7.6$  and  $52.7 \text{ Bq mg}^{-1}$  after 24 and 72 h, respectively). Accordingly, the relative proportion of  $^{14}\text{C}$  in the ethanol–water-soluble compounds decreased. After 72 h, more than one-third of the radioactivity accumulated in the stem was incorporated into the lignocellulosic fraction while the proportion incorporated into suberin represented ~6%.

### Distribution of photoassimilated $^{14}\text{C}$ during the year after labeling

Table 2 shows the values of specific radioactivity measured in cork oak stems and in their chemical fractions during the period comprised between 1 week and 1 year after the plant exposure to  $^{14}\text{CO}_2$ . If these values are compared with the values obtained from the short-term study (Table 1) it is deduced that an important increase in stem specific radioactivity happened in the period comprised between day 3 and day 7 (first week after labeling). One week after labeling most of the stem radioactivity was accumulated in the lignocellulosic components (69.0%) followed by the ethanol and water extractives (22.6%), while suberin contained 7.3% of the total radioactivity. Over the year, there was a decrease in the  $^{14}\text{C}$  proportion in the polar extractives, down to 12.4%, and an increase in suberin (11.2%) and lignocellulosics (75.3%). The lowest proportion of radioactivity (1.0–1.2%) was recorded for the non-polar compounds (waxes and fats).

Suberin was the component with the highest values of specific radioactivity throughout the long-term study. However, at the end of the experiment year the specific radioactivity of suberin decreased somewhat and represented 81% of the mean value recorded for the sampling of week 1. Relatively high values of specific radioactivity were recorded for the ethanol and water-soluble extractives 1

Table 1. Specific radioactivity (Bq mg<sup>-1</sup>) of the chemical components obtained by sequential extraction of cork oak plants sampled at different times between 1 and 72 h after <sup>14</sup>CO<sub>2</sub> photoassimilation. In brackets, relative contribution of each compound (as % of the total radioactivity obtained in each sampling) (*n* = 4 plants per sampling × 2 stem samples per plant). Values followed by the same letter within each column are not statistically different (*P* ≥ 0.05).

Hours after <sup>14</sup> C labeling	Non-polar extractives	Ethanol and water extractives	Suberin	Lignocellulosic fraction	Stem weighted mean
1	0	0.82 ± 0.07 e (100)	0	0	0.17 ± 0.01 d
3	0	10.00 ± 0.59 d (100)	0	0	1.91 ± 0.04 d
8	0.10 ± 0.03 c (1.22)	40.47 ± 1.28 c (91.60)	0.10 ± 0.03 c (1.07)	0.28 ± 0.02 c (6.11)	7.16 ± 0.32 c
24	2.30 ± 0.19 b (1.50)	50.62 ± 0.91 b (83.95)	7.65 ± 0.32 b (2.70)	1.00 ± 0.11 b (11.85)	9.34 ± 0.50 b
72	23.40 ± 1.20 a (3.50)	80.83 ± 2.02 a (54.25)	52.71 ± 0.92 a (5.80)	2.05 ± 0.05 a (36.45)	15.59 ± 0.70 a
<i>P</i>	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2. Specific radioactivity (in Bq mg<sup>-1</sup>) of the chemical components obtained by sequential extraction of cork oak plants sampled at different times throughout the year after <sup>14</sup>CO<sub>2</sub> photoassimilation. In brackets, radioactivity distribution (as % of the total radioactivity obtained in each sampling) among chemical components. (*n* = 4 × 2). Values followed by the same letter within each column are not statistically different (*P* ≥ 0.05).

Weeks after <sup>14</sup> C labeling	Non-polar extractives	Ethanol and water extractives	Suberin	Lignocellulosic fraction	Stem weighted mean
1	55 ± 3 ab (1.06)	114 ± 5 a (22.60)	440 ± 26 (7.32)	67 ± 1 a (69.02)	79 ± 1 a
2	81 ± 11 ab (1.04)	95 ± 3 ab (19.44)	384 ± 40 (7.95)	65 ± 2 a (71.57)	74 ± 1 a
3	82 ± 18 ab (1.06)	61 ± 12 c (16.83)	288 ± 35 (9.60)	51 ± 8 ab (72.51)	57 ± 3 b
4	38 ± 4 b (1.06)	48 ± 9 cd (17.10)	258 ± 25 (9.33)	43 ± 2 ab (72.51)	48 ± 1 c
6	95 ± 6 a (1.07)	71 ± 5 bc (15.61)	377 ± 24 (10.93)	64 ± 5 a (72.39)	73 ± 1 a
8	79 ± 5 ab (1.09)	52 ± 4 cd (13.27)	364 ± 43 (11.89)	50 ± 4 b (73.75)	52 ± 1 bc
26	63 ± 8 ab (1.15)	27 ± 4 e (13.06)	336 ± 46 (11.33)	35 ± 5 b (74.46)	37 ± 1 d
52	38 ± 6 b (1.14)	21 ± 2 e (12.44)	358 ± 53 (11.16)	38 ± 8 b (75.26)	36 ± 2 d
<i>P</i>	<0.001	<0.001	0.063	<0.001	<0.001

Table 3. Radioactivity distribution (as % of the total radioactivity obtained in each sampling) among the chemical components of the lignocellulosic fraction obtained by sequential extraction of cork oak plants sampled at different times after <sup>14</sup>CO<sub>2</sub> photoassimilation (*n* = 4 × 2). Values followed by the same letter within each column are not statistically different (*P* ≥ 0.05).

Weeks after <sup>14</sup> C labeling	Hemicelluloses	Amorphous cellulose	Crystalline cellulose	Lignin	Lignocellulosic fraction
1	23.75 ± 0.95 a	16.59 ± 0.28 c	15.04 ± 0.32 a	13.64 ± 0.50 d	69.02 ± 0.57 d
2	23.79 ± 0.14 a	17.09 ± 0.25 c	14.71 ± 0.28 a	15.98 ± 0.33 c	71.57 ± 0.41 c
3	22.80 ± 0.45 a	19.23 ± 0.24 a	13.76 ± 0.31 b	16.72 ± 0.56 c	72.51 ± 0.50 c
4	21.88 ± 0.33 b	20.43 ± 0.47 a	11.40 ± 0.49 c	18.80 ± 0.64 b	72.51 ± 0.55 c
6	22.87 ± 0.28 ab	18.51 ± 0.54 b	11.84 ± 0.68 c	19.17 ± 0.62 b	72.39 ± 0.58 c
8	21.92 ± 0.25 b	20.13 ± 0.19 a	13.09 ± 0.66 b	18.61 ± 0.89 b	73.75 ± 0.43 b
26	23.56 ± 0.30 a	15.12 ± 0.64 d	12.11 ± 0.46 c	23.67 ± 0.58 a	74.46 ± 0.56 a
52	23.66 ± 0.32 a	16.98 ± 0.65 c	10.88 ± 0.24 d	23.74 ± 0.29 a	75.26 ± 0.16 a
<i>P</i>	0.015	<0.001	<0.001	<0.001	0.001

week after labeling, but these values decreased subsequently. This was the result of their translocation and incorporation in the building up of the structural components while new soluble molecules were formed from non-radioactive photoassimilated carbon. The lipids and other non-polar soluble compounds, which had low specific radioactivity in the first week (12.5% of that of suberin), maintained the trend of low values throughout the year. Regarding the lignocellulosic fraction, the specific radioactivity decreased

significantly (43.3%) throughout the year and this decrease was much higher than that which occurred in suberin. Little variation in the radioactivity distribution among hemicelluloses and amorphous cellulose was recorded (see Table 3) throughout the year; on the contrary, values of crystalline cellulose decreased significantly (from 15.0 to 10.8%). However, the radioactivity proportion in lignin increased substantially, from 13.6% in the first week to 23.7% 1 year after the labeling.



### Allocation of $^{14}\text{C}$ to stem tissues: microautoradiographic study

$^{14}\text{C}$  translocation to stem tissues after  $^{14}\text{CO}_2$  photoassimilation in late spring was monitored by microautoradiography of stem cross sections taken from the young trees at different times during the period from 24 h to 52 weeks after pulse labeling, as shown in Figures 3 and 4. The dark regions in the microautoradiography pictures correspond to the accumulation of radioactive carbon.

The dark ring region in Figure 3I corresponds to the cambium and the intensity of the black zone compared with other dark spots shows that after 24 h the highest accumulation of  $^{14}\text{C}$  occurs in the cambial zone. The structure of the cork oak stem as shown in the picture is similar to the one described by Graça and Pereira (2004). Discontinuous dark spots are also observed within the ring of perivascular fibers corresponding to the primary phloem that also acts as a  $^{14}\text{C}$  sink, although to a much lesser extent than the cambium. It is worth mentioning that no photoassimilated  $^{14}\text{C}$  was present in the periderm at this stage, since no darkening is observed.

Leaf-assimilated  $^{14}\text{C}$  was detected in suber cells 1 week after  $^{14}\text{C}$  photoassimilation. Figure 3II shows that—in addition to the primary phloem that remains dark—two dark spots in the periderm are located on active suber cells that have been formed by the phellogen in the space of 1 week. Dark spots

appear discontinuously in the phellogen around the stem perimeter in all the microautoradiographs taken from 1 week onward (see Figures 3II and 4I–III). Growth of new layers of cork cells can be noticed in microautoradiographs taken 8 weeks after  $^{14}\text{C}$  labeling (see Figure 3III). Six months after labeling (Figure 4II) the layers of  $^{14}\text{C}$ -labeled cork cells have now been pushed into the more external part of the stem by the newly formed layers of cork cells underneath, that are not radioactively labeled. One year after labeling (Figure 3IV) the labeled cork cells of the periderm are located nearer the external part of the stem due to the loss of older cells.

Secondary xylem growth can be noticed from the examination of microautoradiographs taken from 8 weeks onwards. The two-ended arrows in Figure 4 indicate the new layers of secondary xylem that were formed during the period comprised between xylem labeling and sampling date, and that remain undarkened between the previously labeled xylem and the previously labeled secondary phloem. In Figure 4I, due to the radial growth of secondary xylem, the secondary phloem is pushed into the ring of perivascular fibers where the primary phloem is located. Twenty-six weeks after the plant exposure to  $^{14}\text{CO}_2$  (Figure 4II) the secondary xylem growth is more conspicuous than in Figure 4I (a picture taken 8 weeks after  $^{14}\text{C}$  leaf assimilation); a similar observation can be made from 52-week-sampling microautoradiographs (Figure 4III).

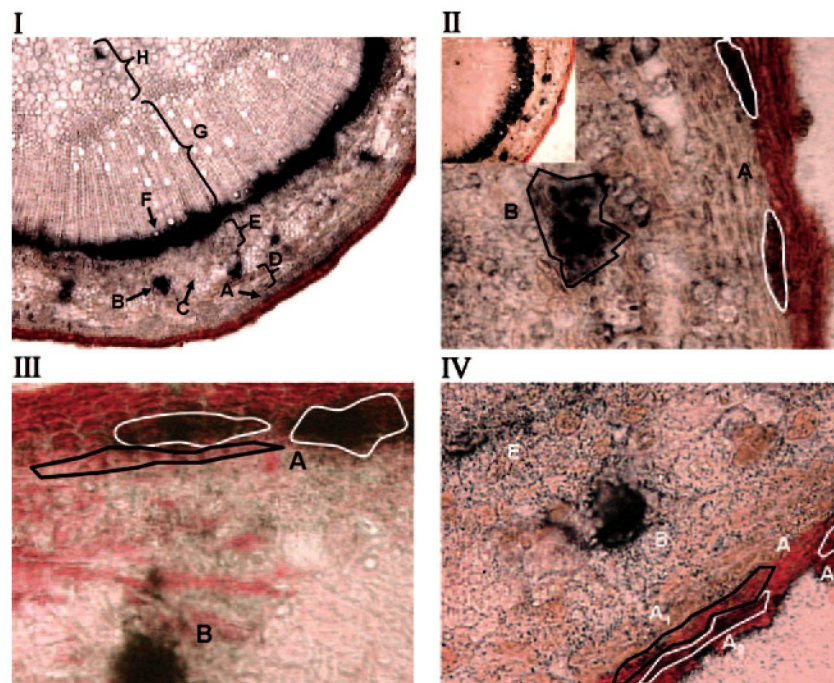


Figure 3. Distribution of  $^{14}\text{C}$  (dark regions in microautoradiographs) in stem tissues. (I) ( $\times 50$ ) Twenty-four hours after labeling the cambial zone accumulates most  $^{14}\text{C}$ ; dark spots (B) can also be observed in primary phloem. (II) ( $\times 100$ ) One week after labeling some cork cells appear labeled (outlined in white). (III) ( $\times 200$ ) Two or three new layers of cork cells (outlined in black) were formed after 8 weeks and appear under the labeled cork cells. (IV) ( $\times 200$ ): After 1 year the labeled cork cells of the periderm are now located nearer the external part of the stem due to the loss of older cells (unlabeled and labeled cells). Underneath, 6–7 layers of unlabeled cork cells were formed. Key to letters identifying tissues: A, periderm; B, primary phloem; C, perivascular fibre; D, cortex; E, secondary phloem; F, vascular cambium; G, secondary xylem; H, pith.



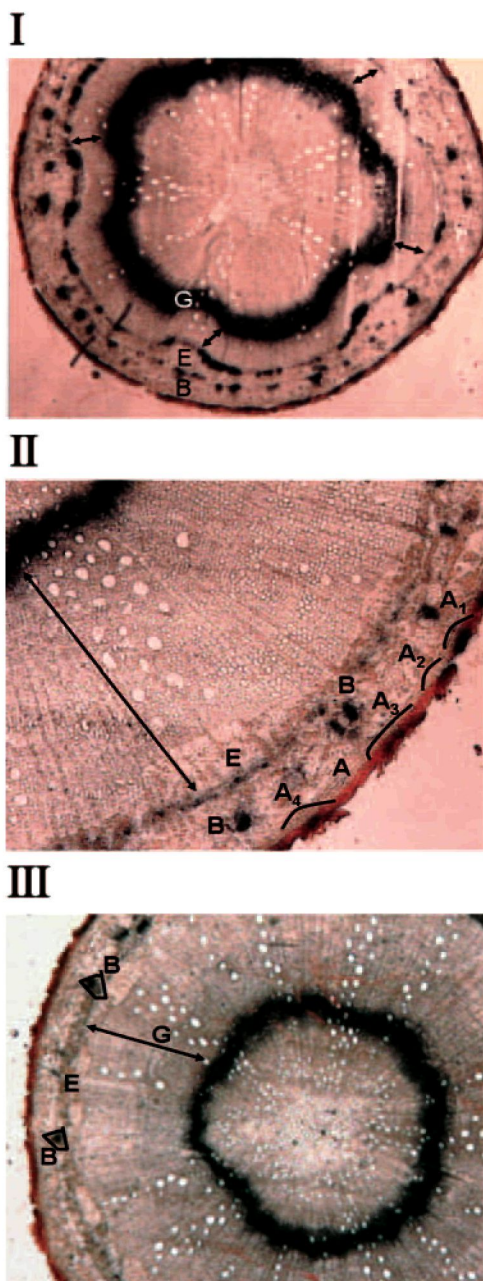


Figure 4. Microautoradiographs showing xylem growth after  $^{14}\text{C}$  labeling. (I) ( $\times 20$ ) The two-ended arrows show the new layers of secondary xylem growth occurring in the first 8 weeks. (II) ( $\times 50$ ) Twenty-six weeks after labeling, xylem growth is more conspicuous and the labeled secondary phloem appears closer to the primary phloem. Layers of labeled cork cells (A1, A2, A3 and A4) are also shown. (III) ( $\times 20$ ) Xylem growth after 52 weeks. Labeled spots of primary phloem are outlined in black. (See legend to Figure 3 for key to letters).

### Quantification of xylem growth

The stem radial growth of cork oak plants in this work can be determined by counting the number of periclinal cellular divisions in the period between  $^{14}\text{C}$  pulse labeling and plant sampling. The average time taken for cellular division during a

particular period can be determined by dividing the corresponding time period by the number of single cell layers formed during that period. The proposed method of xylem growth evaluation can be used only when a sufficient number of unlabeled cell layers is produced that enables the tissues to be differentiated visually. In our work this occurred 3 weeks after  $^{14}\text{C}$  photoassimilation.

Table 4 shows the results of this quantification. The total number of the cambial periclinal cellular divisions during 1 year was 77, equivalent to an annual average cell division time of 4.7 days. In June (periods comprised between labeling and week 3 and between week 3 and week 4), the average time for cell division was 2.3 days. In July it increased to 3.0 days. Then, it went up to 3.6 days (August–November) and finally to 9.2 days (December–May).

### Discussion

Results of the chemical fractioning of young cork oak stem are consistent with data given for *Quercus* and other hardwoods in the literature (Fengel and Wegener 1989, Balaban et al. 1994). The short-term study of  $^{14}\text{C}$  allocation to stem chemical components showed that the  $^{14}\text{C}$  photoassimilated by the leaves started to translocate toward the stems soon after  $^{14}\text{CO}_2$  pulse labeling, which indicates that the labeling time (late spring) coincided with a period of active growth. In fact, labeling time had been chosen taking into account the seasonal progression of phenological events of cork oak reported by other authors (Oliveira et al. 1994). Seasonal changes in the assimilation rate of mature cork oak trees (8.5–9.5 m high) in southern Portugal were reported by Vaz et al. (2010); they found that the maximum rate of photosynthesis attained high values ( $0.204\text{--}0.224\text{ mol m}^{-2}\text{ s}^{-1}$ ) in spring and declined during the dry summer period.

In our study, radioactivity was detected in the stems from the very beginning of the measurements (samplings taken 1 h after  $^{14}\text{CO}_2$  pulse labeling); the first  $^{14}\text{C}$ -labeled fraction was the ethanol and water extractives. In only 8 h after  $^{14}\text{CO}_2$  pulse labeling, other chemical components of the stem—including suberin—were  $^{14}\text{C}$  labeled. The microautoradiographic study also showed the early presence of  $^{14}\text{C}$  in stem tissues. Thus, 1 day after  $^{14}\text{C}$  pulse labeling, radioactivity was allocated to the cambial zone and primary phloem; no dark spots were seen in the periderm region. Cambium was by far the tissue that accumulated most of the stem radioactivity at that time.

Early translocation of recent photoassimilates from leaves to stems has also been noticed in other tree species as a result of  $^{14}\text{CO}_2$  pulse-labeling experiments, like the ones reported by Lockhart et al. (2008) and Kuhns and Gjerstad (1991) for *Quercus pagoda* Raf. and *Pinus taeda* L., respectively. Lockhart et al. (2008) assumed that the 48-h period was sufficient for  $^{14}\text{C}$  translocation; consequently, their study involved plant

Table 4. Xylem growth evaluation at different periods of time after <sup>14</sup>C labeling.

Time since <sup>14</sup> C labeling	3 weeks	4 weeks	8 weeks	26 weeks	52 weeks
Sampling month	June	June	July	November	May
Mean temperature (°C)	24	24	23	10	17.5
Total number of xylem cells produced since <sup>14</sup> C labeling	9 ± 0.46	13 ± 0.58	23 ± 1.00	57 ± 1.39	77 ± 1.79
Average cell division time since <sup>14</sup> C labeling (days)	2.3	2.3	2.7	3.2	4.7
Average cell division time in the period since the previous sampling (days)	2.3	2.3	3.0	3.6	9.2

harvesting after 48 h <sup>14</sup>C labeling. Kuhns and Gjerstad (1991) performed plant samplings 8, 24 and 72 h after <sup>14</sup>CO<sub>2</sub> pulse labeling. In their experiment, they found that sugars were the most heavily labeled fraction; the same conclusion can be drawn from the results of specific radioactivity and radioactivity distribution in our short-term study (72 h) of cork oak.

The fraction of C assimilated by the cork oak sapling that is used by the stem to ensure its growth can be estimated from the values of CO<sub>2</sub> concentration in the chamber air, total radioactivity introduced into the chamber, specific activity and stem weight. In this work the fraction was estimated at 0.1, 11.2 and 57.0% of the leaf-assimilated CO<sub>2</sub> in the first 1 h, 72 h and 1 week after labeling (peak of stem specific activity), respectively. Then, the values became lower, consistent with the fact that the stem specific activity decreased; after 52 weeks, 26.0% of the initial leaf-assimilated CO<sub>2</sub> remained allocated to the stem.

Allocation of <sup>14</sup>C among chemical components and stem tissues changed as time progressed (see Table 5). During the first 72 h most of the stem radioactivity was located in the ethanol and water extractives, which was consistent with the results obtained by Kuhns and Gjerstad (1991). As shown in the Results section, the radioactivity proportion in the ethanol and water extractives decreased after the 3 h sampling. One week after <sup>14</sup>C labeling, most of the radioactivity (69% on average) was found in the lignocellulosic fraction, i.e., in structural components of the stem. Interestingly, 12.4% of the stem radioactivity was found in the ethanol and water extractives after 52 weeks; therefore, a fraction of C assimilated 52 weeks ago was still available as soluble compounds for the sapling's needs.

By using a similar approach to the above-mentioned method, the fraction of leaf-assimilated C allocated to each chemical component can be estimated. According to that approach, 39.3% of the leaf-assimilated C was allocated to lignocellulosic components after 1 week while the amount allocated to suberin represented 4.2%. Thus, translocation to periderm was visualized in the microautoradiographies taken 1 week after <sup>14</sup>C pulse labeling, where some spots of the periderm appeared labeled.

Table 5. Allocation pattern of leaf-assimilated C into the stem: summary of main trends.

Time since C assimilation	Allocation of leaf-assimilated C into the stem
1 h	<ul style="list-style-type: none"> <li>• Presence of leaf-assimilated C in stem, but only in the cambial zone (ethanol and water extractives).</li> </ul>
8 h	<ul style="list-style-type: none"> <li>• Presence of leaf-assimilated C in every chemical component of stem, including suberin.</li> </ul>
24 h	<ul style="list-style-type: none"> <li>• Accumulation of leaf-assimilated C in stem keeps increasing.</li> <li>• Increase in the accumulation rate of suberin.</li> </ul>
3–7 days	<ul style="list-style-type: none"> <li>• Most active period for suberin accumulation.</li> </ul>
1 week	<ul style="list-style-type: none"> <li>• Peak of accumulation of leaf-assimilated C in stem (57.0%).</li> <li>• Estimate of the distribution of leaf-assimilated C to stem chemical components: 0.6% non-polar extractives, 12.9% polar extractives, 4.2% suberin, 39.3% lignocellulosics.</li> <li>• Evidence that phellogen is active—although not in its entire circumference—in late spring.</li> </ul>
2–26 weeks	<ul style="list-style-type: none"> <li>• Stem concentration in C assimilated in late spring decreases, which points to a dilution effect by new assimilates and possible changes in assimilate movement.</li> <li>• Stable values of leaf-assimilated C allocated to suberin (~4%) suggest that its synthesis is mainly realized in late spring.</li> <li>• Growth rate of secondary xylem particularly high in the first 4 weeks after C photoassimilation.</li> </ul>
52 weeks	<ul style="list-style-type: none"> <li>• The fraction of leaf-assimilated C kept by stem is estimated at 26.0%.</li> <li>• Histological evidence that the small decrease in the amount of leaf-assimilated C allocated to suberin is due to loss of cork cells.</li> </ul>

Interestingly, all the microautoradiographs taken from 1 week onward showed that the phellogen was discontinuously labeled. The labeled cork cells were always found in spots. This fact indicates that at the time of <sup>14</sup>C pulse labeling the phellogen was not completely active in its entire circumference—in contrast to the vascular cambium—and that it was differentiating phellem cells only in the dark spots.



Changes in radioactive distribution inside the lignocellulosic fraction were noticed in the long-term chemical study, which could indicate changes in stem tissue composition with time. As a general trend, the radioactivity proportion of hemicelluloses remained rather stable after week 1, while that of lignin increased throughout the year; that fact suggests lignification of stem. The radioactivity proportion of suberin increased as well. However, the results of mass distribution among chemical components of the stem lignocellulosic fraction did not reveal changes in stem composition. This apparent contradiction can be explained by methodological limitations imposed by the weight of young cork oak stems (~8 g dry mass). In addition to that, it highlights the chief advantage of isotopic tracers in this type of study: the ease of detection of extremely small amounts of labeled compounds.

The short- and long-term studies showed that  $^{14}\text{C}$  translocation from leaves to stems was rather fast in our experimental conditions, as it happened mainly in the first week after  $^{14}\text{CO}_2$  photoassimilation. Thus, the specific radioactivity of the stem, ethanol and water extractives, suberin and stem lignocellulosic fraction peaked in the sampling taken 1 week after the  $^{14}\text{CO}_2$  pulse labeling. Compared with previous studies, this finding shows that the chase period considered by other authors after  $^{14}\text{CO}_2$  assimilation by tree species—48 h in Lockhart et al. (2008) for *Q. pagoda*, 72 h in Kuhns and Gjerstad (1991) for *P. taeda* and 72 h in Friend et al. (1991) for *Populus* spp.—would be insufficient for studying C translocation to stems of *Q. suber*. Therefore, the best sampling period to follow the fate of labeled C in cork oak saplings would be 1 week after labeling.

Evolution of suberin specific radioactivity in our experimental conditions suggests that the synthesis of suberin—as well as the synthesis of components of the lignocellulosic fraction—is fast at this time of the year (i.e., in late spring). In order to elucidate the relationship between C assimilation and suberin synthesis, the increase in suberin specific radioactivity per unit of time ( $\text{Bq mg}^{-1} \text{ h}^{-1}$ ) over different periods of time—until the suberin peak time—was calculated. For the sake of convenience, this rate was called ‘relative radioactivity accumulation rate’. Figure 5 shows that the synthesis of  $^{14}\text{C}$ -labeled suberin in cork oak stems started in the period comprised between 3 and 8 h after the  $^{14}\text{CO}_2$  pulse labeling but the most active period for suberin accumulation was the period comprised between 3 and 7 days.

Dilution of  $^{14}\text{C}$  concentration in the stem was noticed from the 2-week sampling onwards, when the values of stem specific radioactivity showed a tendency to go down. This fact was attributed to the synthesis of new molecules from unlabeled carbon that would have been photoassimilated after the  $^{14}\text{C}$  pulse labeling; additionally, mobilization of  $^{14}\text{C}$  towards other plant organs—for instance, roots or new leaves—could also have contributed to this effect. As stated in the review by

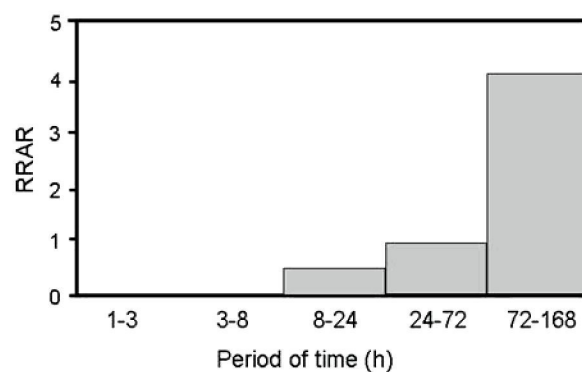


Figure 5. Relative radioactivity accumulation rate of suberin (RRAR,  $\text{Bq mg}^{-1} \text{ h}^{-1}$ ) calculated for different periods of time after  $^{14}\text{CO}_2$  photoassimilation.

Lacointe (2000), the flow of carbon assimilates in trees is a complex and dynamic system; changes in assimilate movement from source areas to sink areas may happen as a result of a number of processes and factors. Using  $^{13}\text{C}$  stable isotope as a tracer, Cerasoli et al. (2004) suggested that the flow of C from source to sink organs operates preferentially within the shortest source–sink distance in 2-year-old cork oak saplings.

Results from this work showed that suberin acted as a sink for the photoassimilated  $^{14}\text{C}$  in the young cork oak. Suberin was by far the fraction with the highest specific radioactivity throughout the year (long-term study), with a maximum value of  $440 \text{ Bq mg}^{-1}$  (sampling taken 1 week after  $^{14}\text{CO}_2$  plant exposure). Second in rank came the ethanol and water extractives, which peaked at  $114 \text{ Bq mg}^{-1}$  (also in the 1-week sampling). Different from other stem chemical components (see Table 2), suberin maintained very high values of specific radioactivity ( $>258 \text{ Bq mg}^{-1}$ ) throughout the experiment duration and the possible effect of  $^{14}\text{C}$  dilution with time was not as apparent as in other stem components.

The fact that suberin specific radioactivity diminished somewhat (it represented 81% of the mean value recorded for the sampling of week 1) at the end of the experiment year could be attributed to both the synthesis of new suberin molecules—that would not be radioactive—and the natural loss of periderm labeled cells. Fractures that usually occur in the periderm because of the radial increase of the plant stem may result in loss of the outermost layers of cork cells. In a histological study of cork oak, Graça and Pereira (2004) found that the initial stage of formation of lenticels starts in the first year of growth; later on, lenticular channels are formed. In our experiment the formation of lenticels would result in a decrease of the specific radioactivity of the stem suberin. Thus, microautoradiographs taken 52 weeks after  $^{14}\text{C}$  pulse labeling showed the loss of labeled cork cells. This finding supports the hypothesis that the small decrease of suberin specific radioactivity is due to the loss of some

labeled cork cells, to some extent. Therefore, it could be assumed that the synthesis of suberin is mainly realized in June—in our experimental conditions—during a short period of the year, which would coincide with the period of most active growth of cork oak. However, further studies—for example, experiments of  $^{14}\text{C}$  labeling at different times—would be needed to prove it.

Time series of microautoradiographs enabled the observation of secondary xylem growth occurring since  $^{14}\text{C}$  pulse labeling and the development of a method of xylem growth evaluation for cork oak. To the best of our knowledge, this is the first reported approach that uses  $^{14}\text{C}$  autoradiography to assess xylem growth of cork oak. Radial growth of *Q. suber* has been investigated by other authors using different methodologies; relevant contributions to this subject are those by: Costa et al. (2002, 2003), who used band dendrometers; Knapic et al. (2007) by means of microdensitometric analysis; and Leal et al. (2008), who applied dendrochronological techniques. All these authors studied the radial growth of *Q. suber* in terms of metric units (mm or cm) whereas in our  $^{14}\text{C}$  tracer study it is studied in terms of cell division time. Results of our microautoradiographic study showed that there was a relation between the time of the year and cell division time, since this was fast in June, but then slowed down more and more throughout the annual cycle. Seasonality of xylem growth shown in this work is in accordance with seasonal values of radial growth of cork oak reported in the literature by using other methodologies (other units). Costa et al. (2002, 2003) found that the maximum radial growth occurred in June–July in the western central part of Portugal. Costa et al. (2002) identified three phases for the diameter growth: (i) early phase in March and April (representing 18.2% of annual growth), (ii) main phase, from May to August (63.9%) and (iii) late phase (September–October, 17.8%). In our work, carried out in the central part of Spain, the maximum growth was recorded in June; later, it declined. The slowing down of cell division after June can be linked on the one hand to the occurrence of high temperatures in summer (Daas et al. 2008), and on the other hand to the subsequent onset of the winter period and the cold (Caritat et al. 2000, Aranda et al. 2005), which greatly influence *Q. suber* metabolism (Chaves et al. 2011).

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## Conflict of interest

None declared.

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